

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
15 July 2004 (15.07.2004)

PCT

(10) International Publication Number  
WO 2004/059277 A2

(51) International Patent Classification<sup>7</sup>:

G01N

(72) Inventors; and

(21) International Application Number:

PCT/IB2003/006203

(75) Inventors/Applicants (for US only): CHAN, Olivia, Wai-Hin [CN/CN]; Kowloon, Hong Kong (CN). CHOW, Tat-Chong [CN/CN]; Shatin, Hong Kong (CN). YU, Vivian [CN/CN]; Kowloon, Hong Kong (CN).

(22) International Filing Date:

24 December 2003 (24.12.2003)

(81) Designated States (national): CN, US.

(25) Filing Language:

English

Published:

— without international search report and to be republished upon receipt of that report

(26) Publication Language:

English

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(30) Priority Data:

60/436,496 26 December 2002 (26.12.2002) US

(71) Applicant and

(72) Inventor: TONG, Sun-Wing [CN/CN]; 9C Carlton Building, Tsimshatsui, Kowloon, Hong Kong (CN).



WO 2004/059277 A2

(54) Title: METHODS OF COLLECTING AND TRANSPORTING VAGINAL DISCHARGE FOR DETECTION OF INFECTIOUS ORGANISMS AND TO FACILITATE CERVICAL CANCER SCREENING

(57) Abstract: The present invention provides a method of testing for the presence of infectious disease agents or host genetic markers comprising applying a device comprising an absorbent and porous material onto the introitus of a female patient; encouraging air drying of at least a portion of the collected vaginal discharge while the device is proximate to the introitus; and determining the presence of infectious disease agents or host genetic markers in the at least partially dried vaginal discharge.

**Methods of Collecting and Transporting Vaginal Discharge  
for Detection of Infectious Organisms and to Facilitate  
Cervical Cancer Screening**

5

**BACKGROUND OF THE INVENTION**

**1. Field of the Invention**

The present invention relates to methods of collection of clinical samples of biological fluids or body discharge. In particular, the invention relates to methods of collection, storage and transportation of vaginal discharge on a simple device, so as to maximize the specimen quantity and quality and to enable the detection of infectious disease agents or host genetic markers by nucleic acid amplification or other technologies.

**BACKGROUND**

**Uterine cervical cancer**

The cervix, or neck of the uterus, is the lower part of the uterus that opens into the upper vagina. It is also the thoroughfare for sperms entering the womb as well as being part of the birth canal. The part of the cervix exposed to the vaginal lumen is usually lined by squamous epithelium, whereas the lining of the lumen of the cervix is glandular. The junction between the two epithelia (transformation zone) is susceptible to infection by HPV (human papillomavirus). This area is also vulnerable to malignant transformation. Common cancer types in this region are squamous carcinoma and adenocarcinoma. Adenosquamous

carcinoma, small cell carcinoma, lymphoma and sarcoma are rare.

Because the cervix is hidden from view, many cancers are at advanced stages when discovered, usually 5 because they cause abnormal bleeding. Dr. George Nicolas Papanicolaou discovered in the 1920s that cells are shed from cervical cancer and when stained on glass slides, reveal themselves to the trained eyes under the microscope. That discovery ushered in early cervical 10 cancer detection by cytological examination. As is now known, most of these cancers are caused by high-risk HPV infection (see below). Detection of HPV is now regarded as capable of giving an even earlier warning, before the cells turn obviously transformed (malignant in 15 appearance).

The American Cancer Society estimates that 12,200 Americans will be diagnosed with cervical cancer in 2003 and up to a third will perish from it. The proportion of women who gets cervical cancer is higher 20 in some societies, e.g. Guanacaste Province in Costa Rica.

#### Papanicolaou smear (the PAP test)

The early detection of cervical cancer has 25 saved many lives in the 50 years since the popularization of the PAP test. The PAP test is based on microscopic interpretation of stained cervical cells that are collected onto glass slides. Through the trained eyes of cytotechnologists and cytopathologists, 30 many early cervical neoplasia cases can be recognized before they turn into invasive cancer.

The conventional PAP test has drawbacks, owing to failure of women to obtain a PAP test, sampling error, poor specimen preparation, subjectivity inherent in any morphological interpretative tests and 5 interpretative error. The conventional Pap smear consequently has false negative rates ranging from 13-70% and false positive of up to 14% (reference 4). The ideal screening test should have as high a sensitivity (100% sensitivity or 0% false negativity) as possible. 10 It is clear that the Pap smear as a cancer screening test is far from being ideal (references 25 & 26). The ThinPrep® Pap Test refined the PAP test by collecting cervical cells into a liquid medium, followed by monolayer preparation on glass slide. The improvement in 15 clarity is translated into more accurate diagnosis. However, women who fail to obtain a PAP test still cannot benefit from this form of cervical cancer screening.

20 Human papillomavirus

The human body plays hosts to microorganisms, which can cause disease. Some of these enter the body through the female lower genital tract, infects the tissue and then other individuals via the same portal 25 through sexual contact or birth.

One of these infectious disease agents is human papillomavirus (HPV). Scientific advances have elucidated the role of this virus in the causation of human cervical cancer. Over 99% of cervical cancers have 30 detectable HPV genetic material. In particular, high risk HPV produces a host of proteins that derail the cell cycle, subvert host immune defense and perhaps take

advantage of the hormonal cycle, to result in malignant transformation of cervical epithelial cells, evasion from elimination by the adaptive immune system of the host, and to enhance its transmission to other 5 individuals.

Examples of such virus-encoded proteins include high risk HPV E7 protein, which is shown to inactivate hypophosphorylated host pRB, thereby preventing it from sequestering E2F, leading to 10 activation of the transcription machinery by E2F and the expression of proteins that push the cell into division. In addition, E7 stimulates the S-phase genes cyclin A and cyclin E, seems to block the function of the cyclin-dependent kinase inhibitors WAF1 (p21 or CIP1) and KIP1 15 (p27), and amplifies the centriole, inducing aneuploidy, all of which contributing to tumorigenesis.

High risk HPVs also produce E6, which mimics cellular MDM-2, and shuttles p53 to the cytoplasmic compartment, where it is destroyed. Without p53, the 20 guardian of the genome, malignant transformation is unchecked. Other transforming properties of E6 include destruction of the pro-apoptotic protein BAK, leading to immortalization of transformed cells, activation of telomerase and possible inhibition of degradation of 25 SRC-family kinases.

Synergistically, E6 and E7 proteins of high-risk HPVs drive the cell into unrestrained proliferation, immortality, aneuploidy and genomic instability, necessary milestones in the malignant 30 transformation of benign cells. Other viral factors, particularly those that subvert the adaptive immune system, interplay with host factors, such as smoking,

mutagens, other infections, immunosuppression, genetic predisposition and several sexual partners, to result in clinical malignancy.

5 HPV testing as an adjunct to or in conjunction with the Papanicolaou smear

Since the association of HPV, in particular high-risk HPV types, with cervical cancer is established, and the molecular mechanisms of cellular 10 transformation and viral evasion of host immune response are being understood, physicians have come to accept HPV testing as an adjunct to the PAP test or for triage of abnormal PAP tests (the Food and Drug Administration [FDA] approved the use of HPV testing in women with 15 abnormal PAP test in March 2000).

Recently, in March 2003, the FDA approved the use of HPV detection as cervical cancer screening in conjunction with the PAP test in women older than 30 years of age 20 (<http://www.fda.gov/bbs/topics/news/2003/new00890.html>).

Specimen collection for HPV testing

Methods of specimen collection have included biopsy, physician directed cervical scrape (similar to 25 the process of obtaining a PAP test specimen), cervico-vaginal lavage and self-obtained specimens utilizing cervico-vaginal lavage, vaginal swab, vulval swab, self insertion and removal of vaginal tampon and urine collection.

30 All of these methods of obtaining clinical specimens have the serious drawback of requiring the patient to present herself to a clinic for the specimen

collection, whether physician-directed or self-obtained. However, one of the major reasons why many women are still presenting with invasive cervical cancer without having a prior PAP test is the psychological barrier of 5 denial, detest of strange environments and strangers, lack of time and other factors such as the unavailability of medical or screening facilities, particularly in developing countries. Other proposals for self-collection involve the deliberation on the part 10 of the patient to purchase an item or to collect one from a clinic, risking stigmatization.

## 2. Description of the Related Art

15 US patent application US2002/0007161 describes the use of a special collection device resembling a sanitary napkin, modified to include a removable sampling strip. Such a device although similar to the regular unmodified sanitary napkin, by virtue of its 20 special nature, has the disadvantage that patients might feel stigmatized (psychologically) when purchasing it, because the act of purchasing or obtaining one is associated with a test for a medical condition. An example is the purchase of over-the-counter male-potency 25 medications, if permitted by the FDA. Retaining the fluid specimen as taught in the art favors survival and continued growth and multiplication of contaminating bacteria and fungi, which produce enzymes that decompose the clinical material of interest, such as viral 30 particles, viral nucleic acids and host genetic markers. The impermeable outer layer of the napkin while preventing soiling of the underwear and/or outside

clothes presents a barrier to rapid evaporation of moisture, contributing to maintaining conditions favorable for bacterial and fungal overgrowth.

Self-obtained cervicovaginal lavage has been 5 advocated as a means of collecting specimens for HPV testing. The method involves insertion of a device into the vagina, followed by the irrigation with a fluid and its collection. The method again involves the purchase of such a device, suffering from the same disadvantage 10 of potential stigmatization as the above example. In addition, some patients find insertion of foreign objects into the vagina offensive.

For the same reason, patients may not like the idea of insertion of vaginal tampons as a means of 15 collecting specimens. This and other methods involving insertion of foreign objects into the vagina are often not acceptable for virgins.

Using other devices, primarily swabs, but also tissue paper, vaginal and vulvar samples can be self- 20 obtained by patients. However, the quantity of sample may not be sufficient for examination in some cases because it involves only a one-time collection. For the same reason, collecting urine may not give the desired sensitivity because while it washes HPV particles from 25 the introitus into the specimen container, the quantity may not be sufficient (explaining the 72% positivity in urine as compared to the 98% positivity in paired cervical swabs; Reference 22).

Thus, a method of specimen collection that 30 does not require the patient to attend a medical clinic, permits the largest amount of vaginal discharge to be collected, does not stigmatize the patient, and also

facilitates the storage, stability, and delivery of the specimen to the laboratory for examination, is needed.

**SUMMARY OF THE INVENTION**

5 The invention provides improved methods for collection, storage and transportation of quantitatively and qualitatively superior clinical specimen from the lower female genital tract on simple devices for the purpose of testing for markers of cervical cancer, such  
10 as HPV, host genetic markers and surrogate markers such as other sexually transmitted disease(s).

**Definitions**

15 "Aneuploid" refers to not having an exact multiple of the haploid number of chromosome.

"Antibody" is a protein (immunoglobulin) that recognizes and binds to an antigen as part of the immune response.

20 "Antigen" means a substance with a molecular surface structure that triggers an immune response, i.e., the production of antibodies, and/or that reacts with (its) specific antibodies (antigen-antibody reaction).

25 "Apoptosis" refers to physiological cell death, or programmed cell death, a process that shapes an organism during development, as well as being a means to prevent viral replication or the propagation of irreparable genetic damage to progeny cells. It is an active process  
30 requiring gene transcription and the expenditure of energy.

"Cell cycle" refers to the sequence of events between mitotic divisions, conventionally divided into G0 (G for gap), G1, S (S for synthesis phase during which DNA is replicated), G2 and M (M for mitosis) phases. Cells that 5 are not dividing are said to be in G0 phase. Cells that transited from G0 to G1 are thought to be committed to completing the cell cycle and dividing.

"Cyclins" are proteins expressed during the cell cycle. 10 Their levels vary markedly during different phases of the cell cycle, unlike their corresponding partner, the cyclin-dependent kinases, which levels do not fluctuate during the cell cycle. The complexes are activated by phosphorylation, and the active kinase then 15 phosphorylates a variety of proteins involved in mitosis, DNA replication, depolymerization of the nuclear lamina, and mitotic spindle formation.

"Cyclin-dependent kinases (CDKs)" are enzymes that are 20 only active when they form a complex with cyclins.

"Cyclin-dependent kinase inhibitors" include two families of proteins, the so-called INK4 inhibitors, comprising p16, p15, p18 and p19, which act on cyclin 25 D/CDK4 and cyclin D/CDK6, and the second group, comprising p21, p27 and p57, which inhibit all CDKs.

"Hybridization" means fusion of two single complementary DNA strands (DNA/DNA hybridization), or 30 the fusion of complementary DNA and RNA strands (DNA/RNA hybridization).

"LOH (loss of heterozygosity)" refers to the loss of an allele. When LOH occurs, inactivation of the remaining allele by mutational inactivation results in complete loss of function of the corresponding gene. If the gene 5 product is a tumor suppressor, then tumorigenesis is not inhibited.

"MDM-2" refers to an oncoprotein that inhibits p53 by binding to the transcriptional activator domain of p53, 10 preventing if from regulating its target. MDM-2 expression is activated by p53 and promotes degradation of p53 by the proteasome.

"Microsatellite" refers to small run (usually less than 15 0.1kb) of tandem repeats of a very simple DNA sequence, usually 1-4bp, for example (CA)<sub>n</sub>.

"Microsatellite instability" refers to a phenomenon characteristic of certain tumor cells, where during DNA 20 replication the repeat copy number of microsatellites is subject to random changes.

"p53" is a tumor suppressor gene product, whose function is protean, and include the arrest of the cell cycle in 25 the event of DNA damage and the induction of apoptosis if the DNA damage cannot be repaired. Because of its central role in preventing tumors, it is found to be inactivated in a little over 50% of human tumors.

30 "Polymerase chain reaction (PCR)" refers to a technique for making many copies of a stretch of DNA sequence in the test tube. It employs repetitive thermal cycling

consisting of denaturation of double-stranded DNA, annealing of appropriate oligonucleotide primers, and extension of the primer by polymerase enzyme.

5 "Polymorphism" means the existence of two or more variants (alleles, phenotypes, sequence variants, chromosomal structure variants) at significant frequencies in the population.

10 "Proto-oncogene (cellular oncogene)" refers to a eukaryotic gene that regulates cell growth or division and when present in truncated form in a retrovirus, behaves as an oncogene, capable of malignant transformation of a eukaryotic cell after integration  
15 into the cell genome.

"Reverse transcriptase" refers to an enzyme complex that occurs in RNA viruses and that can synthesize DNA from an RNA template.

20 "Reverse-transcription PCR (RT-PCR)" refers to the technique for amplification of RNA involving first the synthesis of DNA complementary (cDNA) to a stretch of target RNA employing the enzyme reverse transcriptase,  
25 followed by PCR of the cDNA.

"Single nucleotide polymorphisms (SNPs)" refers to common DNA sequence variations among individuals.

30 "Telomerase" refers to a ribonucleoprotein enzyme that adds nucleotide bases at the telomere.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a photograph of a slab of agarose gel with DNA amplification products separated by electrophoresis, showing the presence of bands specific 5 for HPV in samples of air-dried vaginal discharge and positive control and absence of bands in negative controls.

The agarose gel is stained with ethidium bromide to highlight the amplified DNA under UV-light. 10 The DNA is size fractionated by electrophoresis and shows distinct bands at the predicted location (450 bps), as compared with the 100 bps DNA size ladder at the top (the 100 & 200 bps bands did not show very well in this photograph). Because amplification only occurs 15 with a given set of primers (in this case MY09/11) when HPV is present, the presence of a sharp band at 450 bps is indicative of the presence of HPV in the specimen. Negative controls (clinical samples and water) are negative. Positive control is positive.

20

DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS

According to the invention, the entire process of specimen collection is performed by the patient in the privacy of her own home using self-purchased 25 disposable underwear of any kind of fabrication, preferably absorbent and porous. Alternatively, regular underwear, pantyhose, or a one-time imprint or swab with clean toilet paper are acceptable. Regular clothing is accepted with the understanding that it will be used as 30 a clinical sample and not returnable to the patient.

The soft fabric of the underwear or alternative, herein known as device, moulds onto the anatomical contours of the introitus, facilitating the

transfer of even small amounts of vaginal discharge from the introitus to the absorbent material of the device. A comparatively stiff sanitary napkin may only absorb more copious discharge that drains onto the napkin. If 5 discharge is small in volume, it will preferentially stick to the skin of the introitus rather than adhering to the fabrics of the napkin.

Bacterial and fungal contamination of the underwear or sanitary napkin is inevitable because they 10 may already be present in the vaginal discharge, and skin, and because of the proximity of the introitus to the anus.

Because the fabric of the device is thin and 15 porous, water in the vaginal discharge readily penetrates from the inner surface to the outer surface and escapes into the environment. This natural drying process is facilitated if a skirt, instead of an outside pants is worn. Air-drying thus achieved, is instrumental 20 to the success of the invention, because air-drying of the vaginal discharge stops enzymatic activity of bacteria and host polymorphonuclear neutrophils from breaking down viral particles, viral nucleic acid, host genetic markers and other materials of interest, by the 25 process of decomposition. Air-drying of the vaginal discharge also prevents bacterial and fungal overgrowth.

Addition of anti-bacterial and anti-fungal 30 agents to the device becomes unnecessary. In addition, these additives may be allergenic, inhibitory or complicating subsequent laboratory investigations, or are otherwise unacceptable.

This invention however, does not exclude the use of these additives as an adjunct or prevents the use of special fabric that is inherently inhibitory to bacterial or fungal overgrowth, such as by extracting 5 moisture, thus rendering the secretions dry, or by providing a hostile environment for the growth of microorganisms, yet in no way harming the patient, causing allergic reactions, or compromising subsequent laboratory examination of the specimen.

10 In the morning, the device is worn as if a normal underwear for any period of time, usually until it is changed later in the day. The patient is advised to wear a skirt instead of pants, and not to wear additional undergarment(s) on top of the underwear. At 15 the end of the period, the patient removes the device and puts it into a self-purchased, previously unused, zip-lock plastic bag.

Before or after insertion into the bag, the device if still moist, is dried briefly for about one 20 minute by the gentle cool draft of a consumer-type electric hair drier.

After gently expressing excess air, the zip-lock bag is sealed, without decontamination, addition of preservative, desiccant or additional processing.

25 Next the specimen (device containing vaginal discharge) is inserted into a regular envelope and mailed to the laboratory together with return address (unique identifier but not name, and email, address, and/or telephone number) at the earliest convenience.

30 The patient provides a unique "name" or "identifier" that identifies the specimen, so that reporting can be made anonymously by post, newspaper,

poster, the Internet, or other means. The identifying information should not be the patient's real name but must be unique and known only to the patient.

5 To prevent intentional, ill-willed, grafting of the results on another person, common names are not accepted. Such specimens may be destroyed without testing.

10 On the other hand, specimens that originate from patients who attend medical clinics are allowed to be associated with the real names and contact 15 information of the patients, so that results can be discreetly communicated to them through the health agency.

In the laboratory, the incoming specimens are 15 opened, registered and tested.

Initial preparation of the specimen for DNA extraction  
consists of the following steps:

1. Individually opening the specimen bags in an 20 appropriate biosafety cabinet using decontaminated instruments.
2. Visual identification of the area of the specimen that contains visible stains, which is usually at the most dependent part of the underwear.
- 25 3. Cutting or punching out a full-thickness piece of the stained specimen measuring from 0.2 x 0.2 cm to 1 x 1 cm.
4. Insertion of the procured specimen into an Eppendorf tube followed by DNA extraction.
- 30 5. Optional measurement of the DNA content by spectrometry after extraction, to determine if

dilution is required before subsequent amplification.

6. Dilution, if required of the extracted DNA.

5 For HPV testing, the extracted DNA is processed in the following way:

1. Amplification of HPV DNA from the extracted DNA by the PCR reaction or another method of nucleic acid amplification, using any number of separate reactions employing any number of primer pairs, or in one reaction using a multiplex of primer pairs.
- 10 2. Identification of the amplification product by size fractionation using gel electrophoresis or hybridization (microarray, macroarray, dot blots, line blots, reverse line blots, or antibody-based detection), so as to detect and type the HPV strain(s), if present.

Investigation of host genetic material:

20 For studying markers of malignant or pre-malignant transformation of cellular DNA, the DNA is extracted as mentioned above. The DNA is then subjected to analysis, which include but is not limited to, hypermethylation of promoter sequences of p16 or other tumor suppressors, microsatellite alterations (LOH or instability), amplification of proto-oncogenes, suppression, mutational inactivation or LOH (loss of heterozygosity) of tumor suppressor genes, SNP (single nucleotide polymorphism), such as p53 gene polymorphism, 25 30 or alteration of the pattern of gene expression.

Investigation of host gene expression:

For the purpose of analysis of over-expression of certain mRNA species, such as p16INK4a (encoded by CDKN2A), MCMs (mini-chromosome maintenance) or TERT (catalytic subunit of telomerase), which is over-expressed in most cervical intraepithelial neoplasias, the specimens are extracted for mRNA, which is then reversely transcribed into cDNA and subsequently or simultaneously quantified using a quantitative method of 10 nucleic acid amplification.

Investigation of surrogate markers for cervical cancer:

For the purpose of detecting surrogate markers of cervical neoplasia, the extracted DNA can be analyzed 15 by nucleic acid technology for sexually transmitted infections, such as Chlamydia trachomatis, Neisseria gonorrhoea, Treponema pallidum, Adenoassociated virus, Trichomonas vaginalis, etc.

20

## EXAMPLES

1. Detection of HPV in vaginal discharge.

We were able to detect HPV in air-dried 25 vaginal discharge from 45 samples (100%) obtained from women with histological confirmation of cervical HPV infection or cervical neoplasia.

We extracted DNA from the specimens using the following protocol:

30

1. Add 600 ul Cell Lysis Solution and 3 ul Proteinase K (20 mg/ml). Vortex to mix and incubate at 56°C overnight.
2. Cool sample to room temperature (RT). Remove the specimen using a sterile long 10 ul pipette tip fitted to an autopipette and expel all lysate by pressing it against the inside of the tube.
3. Add 200 ul Protein Precipitation Solution to the cell lysate. Vortex vigorously at high speed for 20 sec. to mix.
4. Centrifuge at 14,000 rpm for 5 min. The precipitated proteins will form a tight pellet. If the protein pellet is not tight, repeat the centrifugation.
5. Pipette the supernatant containing the DNA (leaving behind the precipitated protein pellet, which is discarded) into a clean 1.5 ml microfuge tube containing 600 ul 100% isopropanol. Add 1.0 ul glycogen solution (20 mg/ml, a DNA carrier).
6. Mix the sample by inverting gently 50 times.
7. Centrifuge at 14,000 rpm for 2 min. A white DNA pellet may or may not be visible, depending on the yields. If the DNA yield is expected to be low (<1 ug), increase the centrifugation time to 5 min.
8. Pipette off supernatant as far as possible, a 10 ul pipette is required for final pipetting.
9. Add 300 ul 70% ethanol and invert tube several times to wash the DNA pellet.
10. Centrifuge at 14,000 rpm for 5 min, carefully pipette off supernatant as far as possible, a 10 ul pipette is required for final pipetting. Pellet may be loose, so pipette slowly and watch pellet. With

the cap open, air dry the pellet at room temperature for 15 min.

11. Add 30 ul DNA Hydration Solution.

5 From the extracted DNA, we performed PCR in two separate tubes per specimen, employing different primer pairs:

Primer sequences:

10 Primers GP5+/6+ (reference 23):

PCR primer-GP5+ (5' to 3')

TTTGTTACTGTGGTAGATACTAC

PCR primer-GP6+ (5' to 3')

CTTATACTAAATGTCAAATAAAAG

15

Primers MY09/11\* (reference 24):

PCR primer-MY09 (5' to 3') CGTCCMAARGGAWACTGATC

(forward)

PCR primer-MY11(5' to 3') GCMCAGGGWCATAAYAATGG

20 (reverse)

25 \*Degenerate sequences of the target specific capture probe and hemiprobes shown in lower case letters. The following represent the possible nucleic acids incorporated: r = A or G; y = C or T; m = A or C; k = G or T; w = A or T (A, adenine; C, cytosine; G, guanosine; T, thymidine).

Reaction 1. Primer pair GP5+/6+:

| Master mix reagents       | Volume (ul) |
|---------------------------|-------------|
| 10X Buffer                | 2.5         |
| MgCl <sub>2</sub> (25 mM) | 3.5         |
| Primer GP5+ (10 pmol/ul)  | 2.5         |
| Primer GP6+ (10 pmol/ul)  | 2.5         |

20

|                    |     |
|--------------------|-----|
| DNTPs (10 mM each) | 0.5 |
| Taq (5U/ul)        | 0.1 |
| DW                 | 8.4 |
| Sub-total:         | 20  |
| DNA                | 5   |
| Total:             | 25  |

PCR Conditions:Primer pair HPV GP5+/6+:

Hot start: 94°C 4 mins

5 Denaturation: 94°C 1 min |

Annealing: 40°C 2 mins | X 40 cycles

Extension: 72°C 1 min 30 sec |

Final extension: 72°C 4 mins

Incubation: 4°C until gel electrophoresis

10

Expected size on gel electrophoresis: 140-150 bps.

Reaction 2. Primer pair MY09/11:

| Master mix reagents       | Volume (ul) |
|---------------------------|-------------|
| 10X Buffer                | 2.5         |
| MgCl <sub>2</sub> (25 mM) | 2.5         |
| Primer MY09 (10 pmol/ul)  | 1.25        |
| Primer MY11 (10 pmol/ul)  | 1.25        |
| DNTPs (10 mM each)        | 0.1         |
| Taq (5U/ul)               | 0.1         |
| DW                        | 12.3        |
| Sub-total:                | 20          |
| DNA                       | 5           |
| Total:                    | 25          |

15 PCR Conditions:Primer pair HPV MY09/11:

Hot start: 94°C 5 mins

Denaturation: 94°C 1 min |

Annealing: 55°C 1 min 30 sec | X 40 cycles

Extension: 72°C 1 min |  
Final extension: 72°C 5 mins  
Incubation: 4°C until gel electrophoresis

5 Expected size on gel electrophoresis: 450 bps.

10 After amplification, we perform agarose gel electrophoresis for size fractionation of the PCR product. A representative gel image is shown in figure 1. 1.

15 Early result of our experiments was reported in a peer-reviewed journal (reference 5).

20 Reference 5 discloses the successful detection of human papillomavirus on sanitary napkins. The present invention improves on the disclosed detection method by providing, instead of a regular napkin, a device to collect the vaginal discharge that promotes the air drying of the vaginal discharge while the device is proximate to the introitus. As discussed above, drying of the vaginal discharge reduces or stops enzymatic activity of bacteria and host polymorphonuclear neutrophils from breaking down viral 25 particles, viral nucleic acid, host genetic markers and other materials of interest, by the process of decomposition. Air-drying of the vaginal discharge also prevents bacterial and fungal overgrowth.

30 The following is excerpted from Reference 5 to show the successful detection of HPV from vaginal discharge samples.

Human papillomavirus was successfully detected

by polymerase chain reaction (PCR) in menstrual blood or vaginal discharge collected in sanitary napkins in 100% of 17 women having koilocytosis, cervical intraepithelial neoplasia, or squamous carcinoma.

5 We advocate this form of cervical cancer screening because of its high sensitivity and acceptance by patients. *Diagn. Cytopathol.* 2003;28:140 -141. © 2003 Wiley-Liss, Inc.

10 Testing for cervical human papillomavirus (HPV) infection is being investigated as a means of cervical cancer screening.

1Some investigators have tested for HPV on vaginal tampons2 and have shown high patient acceptance3 and

15 concordance with physician-directed swab.4

We tested the hypothesis of diagnosing genital HPV infection based on the PCR of menstrual blood or vaginal discharge collected on sanitary napkins. We recruited a total of 10 patients, 7 with a pathological 20 diagnosis of HPV infection, 2 with grade 1 cervical intraepithelial neoplasia, and 1 with invasive squamous carcinoma of the cervix.

Soiled intermenstrual or sanitary napkins containing menstrual blood were air-dried by a blower, 25 placed in ziplock plastic bags, and sent to us by regular mail. Small pieces of the napkins were cut out (1 cm x 1 cm x 1 mm) using sterile scissors for direct DNA extraction. We used the same amplification protocol5 for both types of specimens, employing consensus primers 30 GP5<sup>+</sup> and GP6<sup>+</sup> (biopsies and napkins) and, in a different set of reactions (napkins only), primers MY09 and MY11. Gel electrophoresis was performed after 40

cycles of amplification. HPV was detected in the sanitary napkins in 100% (10/10) of cases. Nine specimens were positive using primer set GP5<sup>+</sup>/6<sup>+</sup>. One specimen (case 4) negative with GP5<sup>+</sup>/6<sup>+</sup> tested positive 5 with MY09/11.

Although the number of cases was small, the 100% sensitivity is encouraging. Significantly, even a minimally soiled napkin (case 9) contained sufficient HPV DNA for detection. This study supported the 10 hypothesis that HPV DNA remains detectable in vaginal discharge collected on sanitary napkins up to 7 weeks later and despite contamination by blood.

In populations where the prevalence of HPV 15 infection and/or the rate of Pap testing are low, women can be targeted for Pap test or colposcopy by first testing for HPV status on soiled sanitary napkins. While a negative test is not a complete reassurance, a positive HPV test justifies the trouble, embarrassment, 20 and time of the woman for a Pap test. Patients may even choose to be anonymous when testing for HPV. In this way, more patients may be screened by at least one of the two methods. Adoption of this paradigm shift may extend cervical cancer screening to a larger population 25 of women.

**Note Added in Proof**

Seven additional specimens of intermenstrual soiled napkins were tested with 100% positivity.

References:

1. Schiffman M, Herrero R, Hildesheim A. HPV DNA testing in cervical cancer screening: results from women 5 in a high-risk province of Costa Rica. *JAMA* 2000;283:87-93.
2. Harper DM, Hildesheim A, Cobb JL, Greenberg M, Vaught J, Lorincz AT. Collection devices for human 10 papillomavirus. *J Fam Pract* 1999; 48:531-535.
3. Fairley CK, Chen S, Tabrizi SN, Quinn MA, McNeil JJ, Garland SM. Tampons: a novel patient-administered method for the assessment of genital human 15 papillomavirus infection. *J Infect Dis* 1992;165:1103-1106.
4. Harper DM, Noll WW, Belloni DR, Cole BF. Randomized clinical trial of PCR-determined human papillomavirus detection methods: self-sampling versus 20 clinician-directed biologic concordance and women's preferences. *Am J Obstet Gynecol* 2002;186:365-373.
5. Zehbe I, Wilander E. Two consensus primer systems and nested polymerase chain reaction for human papillomavirus detection in cervical biopsies: a study 25 of sensitivity. *Hum Pathol* 1996;27:812-815.

References:

1. Cannistra SA, Niloff JM. Cancer of the uterine cervix. *N Engl J Med.* 1996 Apr 18;334(16):1030-8.
5. Walboomers JM, Jacobs MV, Manos MM, Bosch FX, Kummer JA, Shah KV, Snijders PJ, Peto J, Meijer CJ, Munoz N. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol.* 10. 1999 Sep;189(1):12-9.
3. Sawaya GF, Brown AD, Washington AE, Garber AM. Clinical practice. Current approaches to cervical-cancer screening. *N Engl J Med.* 2001 May 24;344(21):1603-7.
15. 4. Baldwin P, Laskey R, Coleman N. Translational approaches to improving cervical screening. *Nat Rev Cancer.* 2003 Mar;3(3):217-26.
5. zur Hausen H. Papillomaviruses and cancer: from basic studies to clinical application. *Nat Rev Cancer.* 2002 May;2(5):342-50.
20. 6. Tindle RW. Immune evasion in human papillomavirus-associated cervical cancer. *Nat Rev Cancer.* 2002 Jan;2(1):59-65.
7. Tong TR, Chan OW, Chow TC, Yu V, Leung KM, To SH. 25. Detection of human papillomavirus in sanitary napkins: a new paradigm in cervical cancer screening. *Diagn Cytopathol.* 2003 Mar;28(3):140-1.
8. Human Papillomaviruses: Natural History and Virology.
30. [http://www.fda.gov/ohrms/dockets/ac/01/slides/3805S1\\_02%20Unger/index.htm](http://www.fda.gov/ohrms/dockets/ac/01/slides/3805S1_02%20Unger/index.htm).

9. Nobbenhuis MA, Helmerhorst TJ, van den Brule AJ, Rozendaal L, Jaspars LH, Voorhorst FJ, Verheijen RH, Meijer CJ. Primary screening for high risk HPV by home obtained cervicovaginal lavage is an  
5 alternative screening tool for unscreened women. *J Clin Pathol.* 2002 Jun;55(6):435-9.
10. Harper DM, Noll WW, Belloni DR, Cole BF. Randomized clinical trial of PCR-determined human papillomavirus detection methods: self-sampling  
10 versus clinician-directed--biologic concordance and women's preferences. *Am J Obstet Gynecol.* 2002 Mar;186(3):365-73.
11. Fairley CK, Chen S, Tabrizi SN, Quinn MA, McNeil JJ, Garland SM. Tampons: a novel patient-  
15 administered method for the assessment of genital human papillomavirus infection. *J Infect Dis.* 1992 Jun;165(6):1103-6.
12. Wright TC Jr, Denny L, Kuhn L, Pollack A, Lorincz A. HPV DNA testing of self-collected vaginal  
20 samples compared with cytologic screening to detect cervical cancer. *JAMA.* 2000 Jan 5;283(1):81-6.
13. Sellors JW, Lorincz AT, Mahony JB, Mielzynska I, Lytwyn A, Roth P, Howard M, Chong S, Daya D, Chapman W, Chernesky M. Comparison of self-  
25 collected vaginal, vulvar and urine samples with physician-collected cervical samples for human papillomavirus testing to detect high-grade squamous intraepithelial lesions. *CMAJ.* 2000 Sep 5;163(5):513-8.
- 30 14. Gray RH, Wawer MJ, Girdner J, Sewankambo NK, Serwadda D, Meehan M, Gaydos C, Li C, Quinn T. Use of self-collected vaginal swabs for detection of

Chlamydia trachomatis infection. *Sex Transm Dis.* 1998 Sep;25(8):450.

15. Kailash U, Hedau S, Gopalkrishna V, Katiyar S, Das BC. A simple 'paper smear' method for dry collection, transport and storage of cervical cytological specimens for rapid screening of HPV infection by PCR. *J Med Microbiol.* 2002 Jul;51(7):606-10.

10. Klaes R, Benner A, Friedrich T, Ridder R, Herrington S, Jenkins D, Kurman RJ, Schmidt D, Stoler M, von Knebel Doeberitz M. p16INK4a immunohistochemistry improves interobserver agreement in the diagnosis of cervical intraepithelial neoplasia. *Am J Surg Pathol.* 2002 Nov;26(11):1389-99.

15. Negri G, Egarter-Vigl E, Kasal A, Romano F, Haitel A, Mian C. p16INK4a is a useful marker for the diagnosis of adenocarcinoma of the cervix uteri and its precursors: an immunohistochemical study with immunocytochemical correlations. *Am J Surg Pathol.* 2003 Feb;27(2):187-93.

20. zur Hausen H. Papillomavirus and p53. *Nature.* 1998 May 21;393(6682):217.

25. Storey A, Thomas M, Kalita A, Harwood C, Gardiol D, Mantovani F, Breuer J, Leigh IM, Matlashewski G, Banks L. Role of a p53 polymorphism in the development of human papillomavirus-associated cancer. *Nature.* 1998 May 21;393(6682):229-34.

30. Dong SM, Kim HS, Rha SH, Sidransky D. Promoter hypermethylation of multiple genes in carcinoma of the uterine cervix. *Clin Cancer Res.* 2001 Jul;7(7):1982-6.

21. Nuovo GJ, Plaia TW, Belinsky SA, Baylin SB, Herman JG. In situ detection of the hypermethylation-induced inactivation of the p16 gene as an early event in oncogenesis. *Proc Natl Acad Sci U S A*. 1999 Oct 26;96(22):12754-9.
22. Stanczuk GA, Kay P, Allan B, Chirara M, Tswana SA, Bergstrom S, Sibanda EN, Williamson AL. Detection of human papillomavirus in urine and cervical swabs from patients with invasive cervical cancer. *J Med Virol*. 2003 Sep;71(1):110-4.
23. MV Jacobs, AM de Roda Husman, AJ van den Brule, PJ Snijders, CJ Meijer and JM Walboomers. Group-specific differentiation between high- and low-risk human papillomavirus genotypes by general primer-mediated PCR and two cocktails of oligonucleotide probes. *J Clin Microbiol*. 1995 Apr;33(4):901-5.
24. Yarkin F, Chauvin S, Konomi N, Wang W, Mo R, Bauchman G, Diaz A, Burstein D, Szporn A, Hauptman E, Zhang DY. Detection of HPV DNA in cervical specimens collected in cytologic solution by ligation-dependent PCR. *Acta Cytol*. 2003 May-Jun;47(3):450-6.
25. Franco EL. Are we ready for a paradigm change in cervical cancer screening? *Lancet*. 2003 Dec 6;362(9399):1866-7.
26. Cuzick J, Szarewski A, Cubie H, Hulman G, Kitchener H, Luesley D, McGoogan E, Menon U, Terry G, Edwards R, Brooks C, Desai M, Gie C, Ho L, Jacobs I, Pickles C, Sasieni P. Management of women who test positive for high-risk types of human papillomavirus: the HART study. *Lancet*. 2003 Dec 6;362(9399):1871-6.

CLAIMS

1. A method of testing for the presence of infectious disease agents or host genetic markers comprising: applying a device comprising an absorbent and porous material onto the introitus of a female patient to collect vaginal discharge, either briefly as an imprint or swab, or over a period of time; encouraging air drying of at least a portion of the collected vaginal discharge while the device is proximate to the introitus; and determining the presence of infectious disease agents or host genetic markers in the at least partially dried vaginal discharge.

2. The method of claim 1 wherein the device is a regular or disposable underwear.

3. The method of claim 1 further comprising the step of storing the dried vaginal discharge in a container, without added desiccants, preservatives or other additives.

4. The method of claim 1 wherein the infectious disease agent is human papillomavirus.

5. The method of claim 4 wherein the steps of determining the presence of human papillomavirus comprises nucleic acid amplification and specific identification of the amplified product(s).

6. The method of claim 4 further comprising the step of identifying high-risk, intermediate-risk and low-risk human papillomaviruses.

7. The method of claim 1 wherein the host genomic markers are of malignant or pre-malignant transformation of cellular DNA.

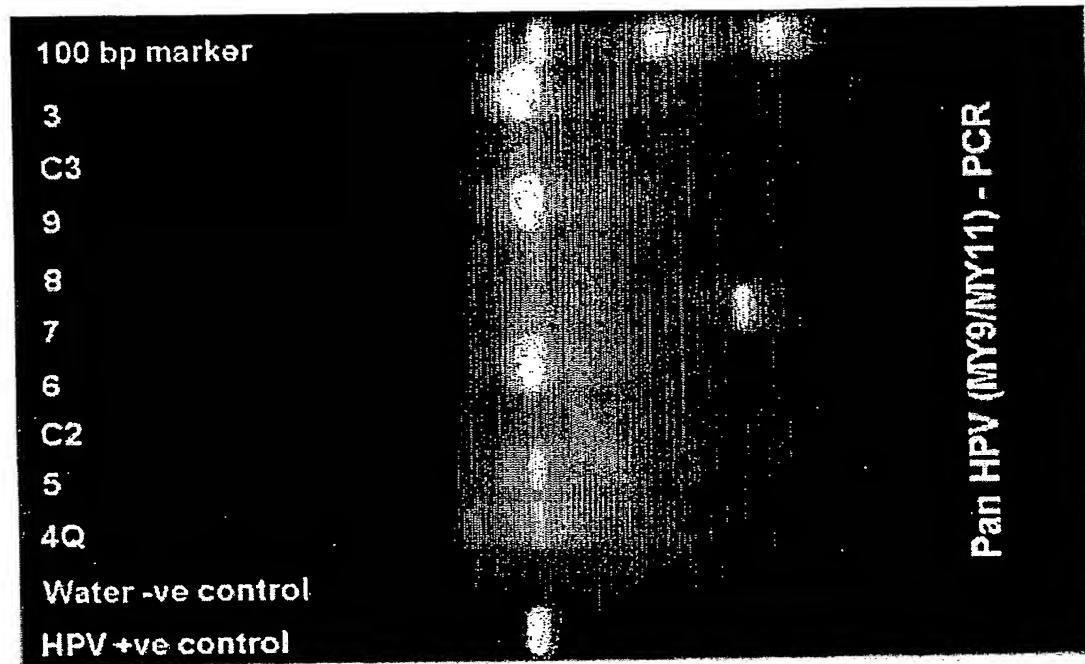
8. The method of claim 1 wherein the host genomic markers are of cellular mRNA.

9. The method of claim 8 where the mRNA is selectively amplified, identified and quantified.

10. The method of claim 1 wherein the infectious disease agents are sexually transmitted disease agents.

5 11. The method of claim 10 where the presence of sexually transmitted disease agents is used as surrogate markers to trigger screening for cervical cancer.

Figure 1..



5

| Label (on photo)  | Legend                    | Result                        |
|-------------------|---------------------------|-------------------------------|
| 100 bp marker     | 100 base pair size marker | First two bands not well seen |
| 3                 | Case 3                    | Positive (strong)             |
| C3                | Negative control 3        | Negative                      |
| 9                 | Case 9                    | Positive (strong)             |
| 8                 | Case 8                    | Positive                      |
| 7                 | Case 7                    | Positive                      |
| 6                 | Case 6                    | Positive (strong)             |
| C2                | Negative control 2        | Negative                      |
| 5                 | Case 5                    | Positive                      |
| 4Q*               | Case 4                    | Positive                      |
| Water -ve control | Water negative control    | Negative                      |
| HPV +ve control   | HPV positive control      | Positive                      |

\* 4Q was initially negative but became positive after using QIAamp Spin Column to purify the DNA.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization International Bureau



(43) International Publication Date  
15 July 2004 (15.07.2004)

PCT

(10) International Publication Number  
WO 2004/059277 A3

(51) International Patent Classification<sup>7</sup>:  
G01N 33/569, A61F 5/455, A61B 0/00

C12Q 1/68.

(72) Inventors; and

(75) Inventors/Applicants (for US only): CHAN, Olivia, Wai-Hin [CN/CN]; Kowloon, Hong Kong (CN). CHOW, Tat-Chong [CN/CN]; Shatin, Hong Kong (CN). YU, Vivian [CN/CN]; Kowloon, Hong Kong (CN).

(21) International Application Number:

PCT/IB2003/006203

(22) International Filing Date:

24 December 2003 (24.12.2003)

(81) Designated States (national): CN, US.

(25) Filing Language:

English

Published:

— with international search report

(26) Publication Language:

English

(88) Date of publication of the international search report:

28 October 2004

(30) Priority Data:

60/436,496 26 December 2002 (26.12.2002) US

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(71) Applicant and

(72) Inventor: TONG, Sun-Wing [CN/CN]; 9C Carlton Building, Tsimshatsui, Kowloon, Hong Kong (CN).

WO 2004/059277 A3

(54) Title: METHODS OF COLLECTING AND TRANSPORTING VAGINAL DISCHARGE FOR DETECTION OF INFECTIOUS ORGANISMS AND TO FACILITATE CERVICAL CANCER SCREENING

(57) Abstract: The present invention provides a method of testing for the presence of infectious disease agents or host genetic markers comprising applying a device comprising an absorbent and porous material onto the introitus of a female patient; encouraging air drying of at least a portion of the collected vaginal discharge while the device is proximate to the introitus; and determining the presence of infectious disease agents or host genetic markers in the at least partially dried vaginal discharge.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB03/06203

## A. CLASSIFICATION OF SUBJECT MATTER

IPC<sup>7</sup> C12Q1/68, G01N33/569, A61F5/455, A61B0/00,

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC<sup>7</sup> C12Q1/68, G01N33/569, A61F5/455, A61B0/00,

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CNPAT, EPOQUE(WPI), NCBI, CA, BA

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages        | Relevant to claim No. |
|-----------|---|-----------------------|
| A         | WO, A1, 9822063, ((PROC ) PROCTER & GAMBLE CO), 28, May, 1998, see the abstract           | 1-11                  |
| A         | WO, A2, 0230266, ((ESAE-N) ESA INC), 18, April, 2002, see the abstract                    | 1-11                  |
| A         | EP, A2, 0617284, ((MOCH ) MOCHIDA PHARM CO LTD), see the abstract<br><i>28. Sep. 1994</i> | 1-11                  |
| A         | BE, A, 881711, ((OKIM-I) OKIMOTO P M), see the abstract <i>30. May 1980</i>               | 1-11                  |

 Further documents are listed in the continuation of Box C.  See patent family annex.

## \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim (S) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search  
13 June 2004(13.06.2004)

Date of mailing of the international search report

08 · JUL 2004 (08 · 07 · 2004)

Name and mailing address of the ISA/CN  
6 Xitucheng Rd., Jimen Bridge, Haidian District,  
100088 Beijing, China  
Facsimile No. 86-10-62019451

Authorized officer



Telephone No. 86-10-62085065

## INTERNATIONAL SEARCH REPORT

Internati.

application No.

PCT/IB03/06203

| Patent document cited in search report | Publication date | Patent family members                           | Publication date                       |
|--|------------------|---|--|
| WO, A1, 9822063                        | 28-05-1998       | US-A-5769834<br>AU-A- 7297698<br>EP -A1-1009346 | 23-06-1998<br>10-06-1998<br>21-06-2000 |
| WO, A2, 0230266                        | 18-04-2002       | US-B1-6548252<br>AU-A-200213164                 | 15-04-2003<br>22-04-2002               |
| EP, A2, 0617284                        | 28-09-1994       | JP-A-6273419<br>US- A - 5552276                 | 30-09-1994<br>03-09-1996               |